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Voegel, Clarissa Daniela ; La Marca-Ghaemmaghami, Pearl ; Ehlert, Ulrike ; Baumgartner, Markus R ; Kraemer, Thomas ; Binz, Tina Maria

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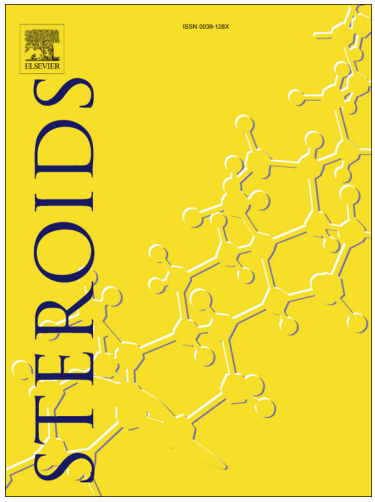

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Steroid Profiling in Nails Using Liquid Chromatography-Tandem Mass Spectrometry

Clarissa Daniela Voegel[a], Pearl La Marca-Ghaemmaghami[b], Ulrike Ehlert[b], Markus R. Baumgartner[a], Thomas Kraemer[c], Tina Maria Binz[a,*]

[a]Center for Forensic Hair Analytics, Zurich Institute of Forensic Medicine, University of Zurich, Zurich, Switzerland

[b]Clinical Psychology and Psychotherapy, Institute of Psychology, University of Zurich, Zurich, Switzerland

[c]Department of Forensic Pharmacology and Toxicology, Zurich Institute of Forensic Medicine, University of Zurich, Zurich, Switzerland

Abstract: The retrospective analysis of endogenous steroid hormones in nails can be used to elucidate endocrine diseases and thus help with their diagnosis and treatment. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) based method was developed for the simultaneous identification and quantification of 12 steroid hormones (aldosterone, cortisone, cortisol, corticosterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, dehydroepiandrosterone (DHEA), 17α-hydroxyprogesterone (17-OHP), dihydrotestosterone (DHT) and progesterone) in human fingernails. Steroid hormones were extracted from 0.5 mg - 10 mg pulverized nail clippings by methanolic extraction, followed by a liquid-liquid extraction. The analysis was conducted with LC-MS/MS in electrospray ionization positive mode. The method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, matrix effect, recovery and robustness. It was successfully applied for steroid profiling in nails of mothers and

their infants where cortisol, cortisone, testosterone, progesterone, androstenedione and 11-deoxycorticosterone could be detected. Furthermore, it could be shown that there is no significant difference in concentrations between left and right hand for cortisol, cortisone and progesterone. A positive linear correlation between cortisol and cortisone in nails was found. In conclusion, it could be shown that nails are a suitable matrix for the retrospective monitoring of cumulative steroid hormone levels.

Keywords: endogenous steroids, liquid chromatography-tandem mass spectrometry, nails, stress

Introduction

Steroid hormone analysis has become an important tool in research on both mental and physical health [1]. The hypothalamic-pituitary-adrenal (HPA) axis is a key regulator in many physiological processes such as physical or mental stress and fatigue. Dysfunction of steroid hormones in the HPA axis can lead to many different endocrinological diseases and syndromes, like Cushing's syndrome [2], Addison's disease [3] and metabolic syndrome [4]. Furthermore disruption of the HPA system has also been associated with mental, immunological and cardiovascular disease which underlines the importance of this system for the human body and accordingly to human and public health [5]. Therefore, the assessment and monitoring of HPA axis steroid hormones is of high clinical importance in the field of endocrinology and psychiatry but also becomes more and more important in the field of neonatology [6]. The classical matrices that are used to monitor steroid hormones are blood, urine and saliva. These matrices underlie the circadian rhythm, which means

concentrations change during the day and therefore show high intraindividual fluctuations. The analysis in these matrices therefore reflects only momentary hormone concentrations at the time of sampling. The use of complementary matrices that can reflect cumulative steroid levels averaged over longer time periods has therefore attracted attention in the last years. The classical and established matrix for the measurement of long-term cumulative steroid levels is the keratinized matrix hair [7-9]. The most prominent hormone that has been analyzed in this context is cortisol which has been shown to be a biomarker for physiological and psychological stress, anxiety and depression [9].

Cortisol and other steroids can also be of interest for prenatal stress research. Numerous studies have found associations between maternal psychobiological stress parameters during pregnancy, neonatal birth outcome, and infant development [10]. The exact underlying mechanisms, however, are still unknown, but cortisol and other steroid hormones seem to play an important role in this regard [10-12]. An excessive exposure on glucocorticoids during gestation is associated with reduced birth weight, higher blood pressure, and increased maternal postpartum depressive symptoms [10]. The fetal stress biology is largely unknown because fetal body fluids are poorly accessible [6]. Therefore, keratinized matrices such as hair and nails can be a valid alternative for fetal stress monitoring, although the hair matrix can be a limitation due to loss of fetal hair close to the birth and more importantly, the late start of hair growth during gestation [6].

Tegethoff et al. showed that one possibility to determine the fetal stress biology is the use of nails as an example of another keratinized matrix [6]. The fetal nail growth starts at the eighth week of gestation and makes it possible to determine in utero

drug exposure [13]. It is also known that steroid hormones are incorporated in the fetal nail during the gestation [6]. Nails are growing at an average rate of 3 mm/month for fingernails [14] . The lower growth rate compared to hair (1 cm/month) allows a more significant accumulation of substances [14]. The incorporation of endogenous and exogenous substances takes place via the nail bed and the nail matrix into the nail tissue [14]. Thus, nails are an interesting matrix for the long-term determination of steroid hormones especially when other matrices are not available.

The focus of this study was to establish a very sensitive LC-MS/MS method that allows the accurate quantification of 12 steroid hormones in human fingernail clippings. As a proof-of-principle authentic nail samples from pregnant and non-pregnant women as well as from infants were measured to show that the method is applicable to quantify a broad spectrum of steroid hormones.

Experimental

Chemical reagents

Aldosterone, cortisone, cortisol, corticosterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, DHEA, 17-OHP, DHT and progesterone (all 1 mg/mL) were purchased from Sigma Aldrich (Buchs, Switzerland). Deuterated internal standards (cortisone-D_7, DHEA-D_5, progesterone-D_9, all 0.1 mg/mL) were purchased from Sigma Aldrich (Buchs, Switzerland). $^{13}C_3$-cortisol and $^{13}C_3$-cortisone (both 0.1 mg/mL) were purchased from Isoscience (USA) and $^{13}C_3$-androstenedione, $^{13}C_3$-DHEA, $^{13}C_3$-progesterone and $^{13}C_3$-testosterone (all 0.1 mg/mL) from Sigma Aldrich (Buchs, Switzerland). Water and methanol were of LC–MS grade (Chromasolv®) and purchased from Sigma-Aldrich (Buchs SG, Switzerland). Acetone, ethyl acetate and ammonium fluoride were purchased from Merck

(Darmstadt, Germany). Reconstitution solution consisted of 970 mL 0.2 mM NH_4F and 30 mL MeOH. All chemicals were of highest analytical grade.

Preparation of standard stock solutions

Standards of each steroid hormone were prepared in methanol at final concentrations of 1 ng/µL. The solutions were further individually diluted with methanol to give working standard solution for each steroid hormone. The internal standard mixture (cortisone-D_7, DHEA-D_5, progesterone-D_9) was prepared to a final concentration of 40 pg/µL. All stock solutions were stored at −20°C until use.

Sample collection and pre-analytics

Nail clippings of infants (n=8) and mothers (n=12) (sampling time during pregnancy and postpartum) were collected by the Institute of Psychology from the University of Zurich (s. Table S1 in Supplementary). This sample collection happened within the assessment phase which was conducted within a larger project on the psychobiological adaptation of women to naturally occurring stress during pregnancy. The entire project was approved by the Cantonal Ethics Committee of Zurich (KEK-ZH-NR: 2012-0408). Nail samples from the left and the right hand of the mothers were collected at different time points (see Table S1 in the Supplementary) and analyzed separately. To ensure enough sample material, all clippings (right and left hand) from the infants were pooled. All samples were washed for 3 min with 2 mL of deionized water, followed by washing for 2 min with the same amount of acetone. The Eppendorf tubes were shaken by hand during the washing process. The washing solutions were decanted and disposed. The samples were dried overnight at room temperature. Subsequently the nail samples were milled with three

steel milling balls in a mixing mill (*Retsch*, Typ MM400). For validation a homogenous pool of nails from different volunteers (that gave oral consent) was prepared by milling the nails for 10 min at 30 Hz with one milling ball (diameter: 20 mm) in the mixing mill and stored at room temperature.

Extraction

For extraction 10 mg nail sample was weighed in a 2 mL Eppendorf tube, pulverized as described above, and 50 µL IS (40 pg/µL cortisone-D_7, , DHEA-D_5, progesterone-D_9) and 1 mL methanol were added. The samples were briefly shaken and placed in a sonication bath for 2 hours at 55 °C for extraction. After centrifugation for 5 min at 9000 g, the methanolic extract was transferred into pill glasses and dried under nitrogen at 35 °C. After the methanolic extraction, three different sample clean ups were tested (method A-C). For method A, a liquid-liquid extraction (LLE) was carried out by adding 1.5 mL ethyl acetate and 220 µL of water to the dried extract. The resulting mixture was subsequently shaken for 10 min. The samples were centrifuged for 5 min at 9000 g and then placed in a freezer (-20 °C) for approximately 60 min. The upper ethyl acetate layer was poured off and dried under nitrogen at 35 °C. For method B, the dry residue was resuspended using 1 mL 10% MeOH. The resuspended extract was subsequently cleaned by solid-phase extraction (SPE) using Oasis HLB columns (Waters, Baden, Switzerland). The columns were conditioned with 1 mL of MeOH followed by 1 mL water. Subsequently, samples were loaded with the resuspended extract (1 mL), washed with 1 mL 30% LC-grade MeOH in water and eluted twice with 300 µL MeOH. Samples were evaporated to dryness at 35 °C under nitrogen. For method C, another SPE was performed with a Biotage Evolute® Express ABN column (Biotage,

Uppsala, Sweden). The sample was diluted in 500 µL MeOH and 1 mL 2% formic acid. The diluted extract (1.5 mL) was loaded onto the column, washed with 1 mL water/methanol, 95/5 (v/v) and eluted with 500 µL MeOH. Samples were evaporated to dryness at 35 °C under nitrogen. All samples were resuspended in 150 µL MeOH and 350 µL reconstitution solution and centrifuged (5 min, 9000 g) before transferring them into LC-MS/MS vials.

LC-MS/MS Analysis

The analysis of the samples was carried out with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) device. The separation was done with a Prominence UFLC system (Shimadzu, Kyoto, Japan) by injecting 10 µL of the samples onto a Phenomenex® Kinetex® XB-C_{18} (2.6 µm, 50 x 2.10 mm) column. The mobile phase consisted of 0.2 mM NH_4F in water/methanol 97/3 v/v (A) and 0.2 mM NH_4F in water/methanol 3/97 v/v (B). The flow rate was 0.45 mL/min and the temperature of the column oven was set to 40 °C. The gradient was as follows: 0–40% B for 0–0.1 min, 40–50% B from 0.1–5 min, isocratic 50% from 5 to 8 min, 50–90% B from 8–11 min, isocratic 90% B from 11 to 14 min, 90–40% B from 14 to 15 min followed by an equilibration step of 1 min. The mass spectrometer, a QTRAP® 6500+ linear ion trap quadrupole mass spectrometer (Sciex, Darmstadt, Germany) was equipped with an electrospray ionization (ESI) source. The quantification was achieved by using the mass spectrometer in multiple reaction monitoring mode (MRM) with an ion spray voltage of 5500 V. Steroid hormones were measured in positive electrospray ionization mode. The parameters of the optimized method used for validation and the analysis of the authentic samples are listed in Table 1. The curtain gas was fixed at 20 psi, the collision gas was set to medium, the ion source

gases 1 at 70 psi and ion source gas 2 set at 50 psi and the source temperature was set to 450 °C. Analyst® software (version 1.6.3, *Sciex*, Darmstadt, Germany) was used for instrument control and data analysis.

Table 1: MRM transitions and MS parameters for all analytes and the internal standards. For each substance, the most sensitive transition was used for quantitation (quantifier) and the second one was used for confirmation (qualifier). Quantifiers are highlighted. DP = declustering potential, EP = entrance potential, CE = collision energy, CXP = cell exit potential, RT = retention time.

Analyte	RT	Q1 Mass [Da]	Q3 Mass [Da]	DP [V]	EP [V]	CE [V]	CXP [V]
Aldosterone 1	1.8	361.0	315.2	31	10	9	6
Aldosterone 2	1.8	361.0	224.9	31	10	23	8
Cortisone D_7	2.3	368.0	169.0	76	10	37	6
Cortisone 1	2.4	361.0	163.0	76	10	31	8
Cortisone 2	2.4	361.0	121.1	76	10	37	6
Cortisone $^{13}C_3$ 1	2.4	364.0	166.0	76	10	31	8
Cortisone $^{13}C_3$ 2	2.4	364.0	124.1	76	10	37	6
Cortisol 1	2.7	363.1	121.0	56	10	29	18
Cortisol 2	2.7	363.1	327.1	56	10	29	18
Cortisol $^{13}C_3$ 1	2.7	366.1	124.0	56	10	29	18
Cortisol $^{13}C_3$ 2	2.7	366.1	330.1	56	10	29	18
Corticosterone 1	4.6	347.0	214.9	56	10	21	12
Corticosterone 2	4.6	347.0	121.3	56	10	21	14
11-Deoxycortisol 1	4.6	347.0	97.1	66	10	21	14
11-Deoxycortisol 2	4.6	347.0	109.0	66	10	9	18
Androstenedione 1	5.6	287.0	97.0	81	10	27	12
Androstenedione 2	5.6	287.0	109.2	81	10	31	6
Androstenedione $^{13}C_3$ 1	5.6	290.1	100.2	61	10	31	16
Androstenedione $^{13}C_3$ 2	5.6	290.1	112.0	61	10	33	12
11-Deoxycorticosterone 1	6.5	331.0	97.0	56	10	27	12
11-Deoxycorticosterone 2	6.5	331.0	109.3	56	10	81	18
Testosterone 1	6.5	289.0	97.0	66	10	29	14
Testosterone 2	6.5	289.0	109.0	66	10	31	16
Testosterone $^{13}C_3$ 1	6.5	292.1	100.0	91	10	27	14
Testosterone $^{13}C_3$ 2	6.5	292.1	112.0	91	10	31	16
DHEA D_5	7.0	293.9	258.0	66	10	29	14
DHEA 1	7.1	288.8	271.0	61	10	11	16
DHEA 2	7.1	288.8	253.0	61	10	13	14
DHEA $^{13}C_3$ 1	7.1	292.1	274.0	36	10	17	18
DHEA $^{13}C_3$ 2	7.1	292.1	256.0	36	10	25	8
17OHP 1	7.7	331.0	97.0	46	10	11	6
17OHP 2	7.7	331.0	109.2	46	10	11	6
DHT 1	9.3	291.0	273.2	61	10	17	14
DHT 2	9.3	291.0	255.4	61	10	17	14
Progesterone D_9	10.4	324.1	100.0	66	10	29	16
Progesterone 1	10.5	315.1	97.0	66	10	27	14
Progesterone 2	10.5	315.1	109.0	66	10	27	14
Progesterone $^{13}C_3$ 1	10.5	319.0	100.0	26	10	29	16
Progesterone $^{13}C_3$ 2	10.5	319.0	112.1	26	10	31	12

Statistics

Statistical analysis was conducted with GraphPad Prism 7.0 (*GraphPad* Software, San Diego, CA, USA). Shapiro Wilk normality test revealed that the data were not normally distributed probably due to the small sample size. A non-parametric Wilcoxon matched-pairs signed rank test was used to estimate the association of left hand and right hand hormone levels and an unpaired Mann-Whitney-Test was used for the association of hormone concentrations between mothers and infants. For correlation analysis Spearman correlation was used. *P*-values > 0.05 were considered as not statistically significant (ns); $p < 0.05$ (*) as significant; $p < 0.01$ (**) as very significant; $p < 0.001$ (***) and $p < 0.0001$ (****) as extremely significant.

Validation

The validation was performed in adaption to the guidelines of the GTFCh appendix C for hair [15]. The quantification and validation of endogenous substances like e.g. steroid hormones is more complex than the one of xenobiotic compounds because often it is not possible to obtain an analyte-free (blank) matrix. If there is no analyte-free matrix available, several approaches can be used for solving this problem. One method is the use of standard addition. Furthermore, there is the possibility of using a surrogate matrix or a surrogate analyte [16, 17].

In our study the use of standard addition was not possible as it requires a large amount of sample material which cannot be provided in nail analysis (typically 1-10 mg nail from one clipping). Also the use of surrogate matrix is not possible as there is not such a matrix for hard keratinized tissue. This is more common when using liquid matrices like blood or saliva.

For this study, a validation was used with authentic matrix (a homogenized nail pool).For the 6 analytes aldosterone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-OHP and DHT the concentration in the nail pool was sufficiently lowso that spiking theauthentic matrix with authentic analytes could perforemd. For the analytes cortisone, cortisol, androstenedione, testosterone, DHEA and progesterone, the approach of using a surrogate analyte was applied as described previously for measuring cortisol in hair [18]. Six stable isotope-labeled standards were chosen as surrogate analytes ($^{13}C_3$-cortisone, $^{13}C_3$-cortisol, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone, $^{13}C_3$-DHEA and $^{13}C_3$-progesterone). The following parameters were tested: response factor (for the surrogate analytes), linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, matrix effects, recovery and robustness. The response factor was calculated by the ratio of the responses found for surrogate and authentic analyte. If the response factor is not 1 it is incorporated into the regression line of the calibration curve in order to get the correct result for the quantification of the endogenous analyte. The LOD is defined as the lowest concentration at which signals were 3 times higher than the background noise for both primary and secondary transitions. The LOQ is the lowest concentration where the signal-to-noise ratio has to be at least 10:1. For linearity, six calibrators with increasing concentrations and a zero sample were prepared (s. Table S3). For accuracy and precision, duplicates of a nail pool with different end concentrations according to substance classes were prepared (s. Table S5). For the $^{13}C_3$-labeled analytes 4 quality controls were prepared with different end concentrations. For $^{13}C_3$-cortisone, $^{13}C_3$-cortisol, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone and $^{13}C_3$-progesterone the end concentrations were 1.2 pg/mg (QC low-1), 6 pg/mg (QC low-2), 40 pg/mg (QC medium) and 400 pg/mg (QC high. For $^{13}C_3$-

DHEA the end concentrations were 120 pg/mg (QC low-1), 240 pg/mg (QC low-2), 300 pg/mg (QC medium) and 400 pg/mg (QC high). For 11-deoxycorticosterone the end concentrations were 6 pg/mg (QC low), 40 pg/mg (QC medium) and 400 pg/mg (QC high). For aldosterone, corticosterone and 11-deoxycortisol the end concentrations were 200 pg/mg (QC low), 400 pg/mg (QC medium) and 800 pg/mg (QC high). For 17-OHP and DHT the end concentrations were 240 pg/mg (QC low), 300 pg/mg (QC medium) and 400 pg/mg (QC high). The measurements were carried out on six consecutive days. The bias as well as the intra- and inter-day precision was calculated for each analyte in each matrix (s. Table S5). For matrix effect and recovery, five replicates from different individuals at low and high concentration levels (Table S6 in the supplementary data) were analyzed. For the matrix effect, the ratio of peak areas of spiked nail (A) to spiked solvent (B) at the same concentration was compared. (Matrix effect = (A/B) x 100). For recovery, the ratio of peak area of spiked matrix before (C) and after (D) extraction was compared. (Recovery = (C/D) x 100). For robustness, six replicates of an authentic nail pool were analyzed and the mean value of detectable hormones plus the relative standard deviation was determined. For a second experiment, a nail pool was analyzed with different sample weight (1 mg, 2 mg, 4 mg, 7 mg, 10 mg, 20 mg) to evaluate if the sample amount has an influence on the concentration.

Results and Discussion

Method development

For an optimal method development, the extraction efficiency was evaluated first. Therefore, three different extraction methods (A-C) were tested and the peak areas were compared. The LLE (A) was superior to the SPE (B-C) in terms of extraction

efficiency and was chosen as clean-up method after the incubation in methanol (data not shown). The LC-MS/MS method was used in positive ESI MRM mode. A representative chromatogram of a hormone mix containing all 12 steroids is shown in Fig. 1. Separation of all 12 steroid hormones was achieved in a total run time of 12 min with good selectivity and sensitivity. So far, there are only very few publications that describe the measurement of multiple steroid hormones in keratinized matrices (hair or nails) with LC-MS/MS which is a more sensitive and advanced method than immunoassay. Gao et al. [19] published a method about the quantitative analysis of 7 steroid hormones (cortisol, cortisone, androstenedione, corticosterone, testosterone, DHEA and progesterone) in human hair and Noppe et al. [7] quantitated 6 steroid hormones in hair (cortisol, cortisone, testosterone, 17-OHP, androstenedione and dehydrepiandrosterone-sulfate (DHEAS). In nails, there are only two publications that describe the quantitation of steroid hormones with LC-MS/MS [5, 20]. To the best of our knowledge, our study describes the first LC-MS/MS method for the simultaneous quantitation of 12 steroid hormones in human nails.

Validation

A validation was performed for the $^{13}C_3$-labeled surrogate analytes ($^{13}C_3$-cortisone, $^{13}C_3$-cortisol, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone, $^{13}C_3$-DHEA and $^{13}C_3$-progesterone) and for the non-labeled analytes (aldosterone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-OHP and DHT) in authentic matrix. When using the surrogate analyte method, first it has to be shown that the surrogate analyte and the authentic analyte elute at the same retention time which was fulfilled

for all the analytes (see Table 1). Additionally the response factor was determined for every surrogate analyte (Table S2). Theoretically, the ideal response factor would be 1. For some analytes the response factors were not equal so the regression line of the calibration curve was corrected by the response factor. The discrepancy of the response factors can be explained by the slightly different chemical characteristics ($^{13}C_3$-label) of the surrogate analyte which might influence their ionization in the mass spectrometer. After verification of the surrogate analytes, validation was performed by spiking authentic matrix with the surrogate analytes. It was assumed that they behave equal to the authentic analytes. Furthermore validation was also performed by spiking analyte-free matrix (nail pool) with aldosterone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-OHP and DHT. A summary of the calibration curves of all analytes is provided in Table S3 showing that good linearity was achieved for all analytes (correlation coefficient > 0.97). The LOQ for $^{13}C_3$-cortisol and $^{13}C_3$-cortisone (1 pg/mg) in this method was lower compared to previously published methods about steroid quantitation in nails [5] which demonstrates the sensitivity of the method. For $^{13}C_3$-DHEA the LOQ (100 pg/mg) was higher than the previously found LOQ (50 pg/mg) [5]. This might be due to the fact that the method presented here involved 12 steroid hormones and 7 surrogate analytes that can be measured simultaneously which might reduce sensitivity of some analytes compared to methods that measure fewer analytes. The results for accuracy are summarized in Table S5 in the supplementary. The results for accuracy and precision met the required criteria. The results of the recovery were found in an acceptable range above 50 % for all analytes as well as the surrogate analytes (Table S6 in the Supplementary). Ion suppression was observed for all analytes but

was still in the acceptable range of 75-125 %. Consequently all validation parameters were fulfilled for the surrogate and non-labeled analytes.

Six replicates of a homogenous and authentic nail pool were measured. Cortisol, cortisone, testosterone, progesterone and androstenedione could be detected in the authentic pool. The mean and the relative standard deviation were calculated and were in an acceptable range below 30 % (cortisol 3.2 pg/mg ± 24.2 %, cortisone 8.7 pg/mg ± 16.8 %, testosterone 1.1 pg/mg ± 26.1 %, progesterone 3.4 pg/mg ± 23.4 %, androstenedione 1.3 pg/mg ± 13.0 %). The measured concentrations of cortisol, cortisone and progesterone in a homogenized pool with increasing sample weight are shown in Fig. 2. The concentration of the hormones in the nail pool were stable and did not differ significantly even if a small sample amount was measured which proves that the weight of sample does not have an influence on the quantitation. Hence, the robustness of the method was proven, and samples with little weight can be measured precisely. This is important when larger cohorts are analyzed because it is not always possible to obtain a large sample amount.

Authentic nail samples

To prove that the developed method is applicable for the measurement of steroid hormones in nails, 31 right and left hand nails from mothers (n=12) (before and after birth) and 8 samples from infants were analyzed (Table S1 in the supplementary).

In Table 2, the mean values in nails of infants and mothers are displayed. The following steroids were detectable in the nails: cortisol, cortisone,, progesterone, testosterone, androstenedione, and 11-deoxycorticosterone. To the best of our

knowledge, this is the first time that the detection of progesterone, androstenedione and 11-deoxycorticosterone in nails was successfully conducted. Previous studies have described the measurement of cortisol, cortisone, DHEA and DHEA-S as well as testosterone [5, 20]. The concentrations of cortisol, cortisone as well as testosterone were in a similar range to previously published concentrations which proves the accuracy of the method [5, 20].

Table 2: Mean steroid hormone concentrations in nails. N shows the number of samples.

Hormone	**Infants** **Mean, Median (range) [pg/mg]**	**Mothers** **Mean, Median (range) [pg/mg]**
Cortisol	3.9, 3.7 (1.5-7.9), N=7	4.5, 4.3 (1.2-12.9), N=31
Cortisone	26.7, 17.9 (12.8-55.6), N=7	17.5, 18.9 (6.2-34.3), N=31
Progesterone	7.5, 5.0 (3.3-16.9), N=7	24.3, 22.0 (1.3-65.9), N=31
Testosterone	< LOQ	1.1, 0.8 (0.4-2.8), N=11
Androstenedione	< LOQ	5.7, 6.8 (1.1-15.0), N=13
11-Deoxycorticosterone	< LOQ	8.5, N=1

Moreover, the comparison of steroid concentrations of cortisol, cortisone, and progesterone (which were the main steroids detected in fingernails of mothers and infants) showed no significant concentration difference in nails of infants compared to their mothers. When comparing steroid levels of mothers during pregnancy and post-partum a trend for slightly higher cortisone values during pregnancy could be seen (Mann-Whitney-Test, $p < 0.05$). For cortisol and progesterone no significant difference could be found. Further interpretations would require more samples to verify if there are differences of steroid levels in infants and adults.

The steroid levels in left and right hand showed no significant differences (paired Wilcoxon matched-pairs signed rank test) for cortisone, cortisol, and progesterone. A good linear relationship was found between left and right hand for each of these hormones (Spearman correlation coefficients, Table 3). Similar results for cortisol have been reported in previous studies [20]. This indicates that sample collection is

not limited on one hand but can be done on either hand on the same fingernail. This can be an important advantage for studies with large sample sizes, especially if not enough material can be provided. It is known that the incorporation of substances into the nail matrix depends on the lipophilicity and the affinity for keratin [20]. The strong binding of steroid hormones to keratin leads to stable concentrations in fingernails of both hands [20]. Cortisol, cortisone and progesterone are rather lipophilic, so it is likely that they bind strongly to keratinwhich explains their good detectability in nails.

Table 3: Mean steroid hormone concentrations in left and right hand and the correlation between left and right hand. Number of pairs = 15.

Hormone	Left hand [pg/mg]	Right hand [pg/mg]	Spearman r
Cortisol	4.7	4.5	0.92 (p< 0.0001)
Cortisone	18	17	0.78 (p< 0.001)
Progesterone	25	24	0.93 (p< 0.0001)

Correlations between cortisol and cortisone concentrations in human fingernails were analyzed by Spearman correlation coefficient. As shown in Fig. 3, a positive linear correlation between cortisol and cortisone levels in nails was found (Spearman r = 0.89, $p < 0.0001$). This indicates the increased conversion from cortisol to cortisone. The key enzyme in the intracellular conversion of cortisol into cortisone and vice-versa is 11β-hydroxysteroid dehydrogenase [21]. The ratio measurement is used in stress related research, because 11β-hydroxysteroid dehydrogenase enzyme activity is altered under stress [22]. The same result has been found for hair cortisol and cortisone which strengthens our result [23].

In this study, we showed that is possible to accurately determine low endogenous steroid concentrations in fingernails and draw conclusions on the steroid metabolism of different individuals. This is especially useful in the research area of steroid hormone metabolism and endocrinology. Furthermore, the sampling of fingernails is non-invasive and can be done at home by the participants. That makes fingernails

an interesting alternative matrix especially if other matrices are not available. Steroid nail analysis has the potential to be clinically used as early indicator of endocrine disorders and fetal stress reactions. It could help to identify Cushing's syndrome and adrenal insufficiency in clinical applications as well as measure chronic stress in many experimental situations. The influence of other diseases like metabolic and sleep disorders on steroid levels in nails still has to be evaluated. Overall, it was shown that the quantitation of steroid hormones in nails is a promising non-invasive method for many research and clinical applications.

Limitations

The presented study is subject to some limitations. First of all, the included infants and their mothers represent a small cohort. No additional information about the samples was accessible so that potential factors that could affect steroid levels could not be evaluated. Furthermore no male subjects are included in the study.

Despite these limitations, the present study gives first insights into steroid levels in nails and the data show trends that are similar to results published in literature. More systematic studies including bigger cohorts should be analyzed in the future to get a more detailed knowledge about steroid levels in nails.

Conclusion

A highly selective and sensitive LC-MS/MS method for the quantification of 12 steroid hormones (aldosterone, cortisone, cortisol, corticosterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, DHEA, 17-OHP, DHT, progesterone) in nails was successfully established. Using a surrogate analyte approach, the method development and validation showed that the use of $^{13}C_3$-labeled steroid hormones is a valid method in case of lack of analyte-free matrices.

Furthermore, it was applied for quantitation of steroid hormones in nails of mothers and their infants. In this study, it was shown that six steroid hormones were quantifiable in nails giving new insights to the steroid metabolism. To the best of our knowledge, this was the first time that progesterone, androstenedione, and 11-deoxycorticosterone were successfully measured in nails. We showed that fingernail clippings can be used for retrospective measurement of steroid hormones in infants and adults which can be helpful in the field of hormone research.

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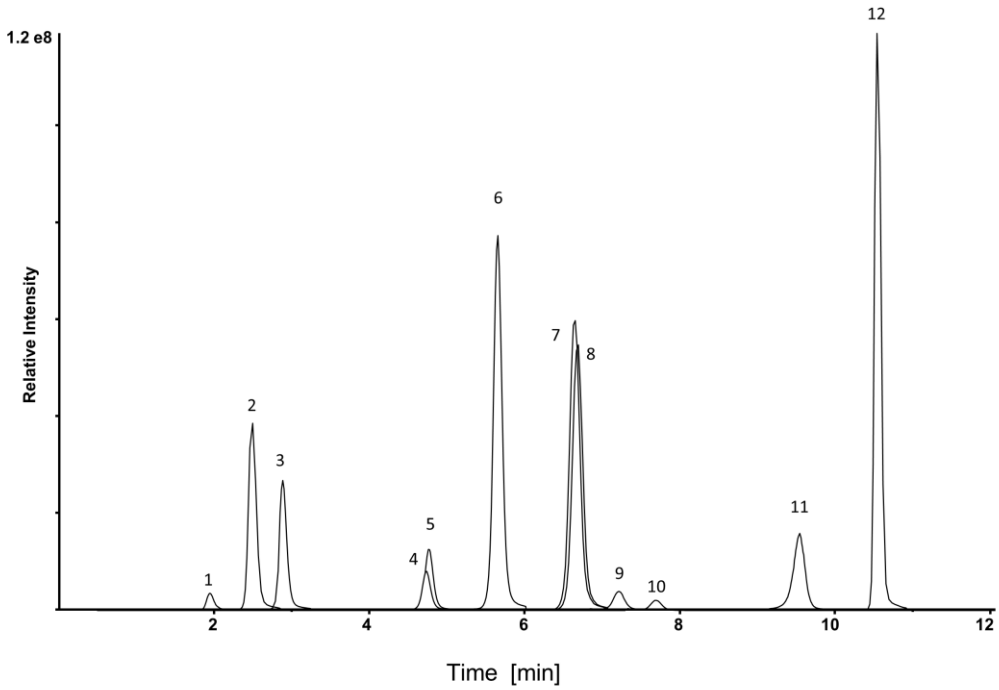

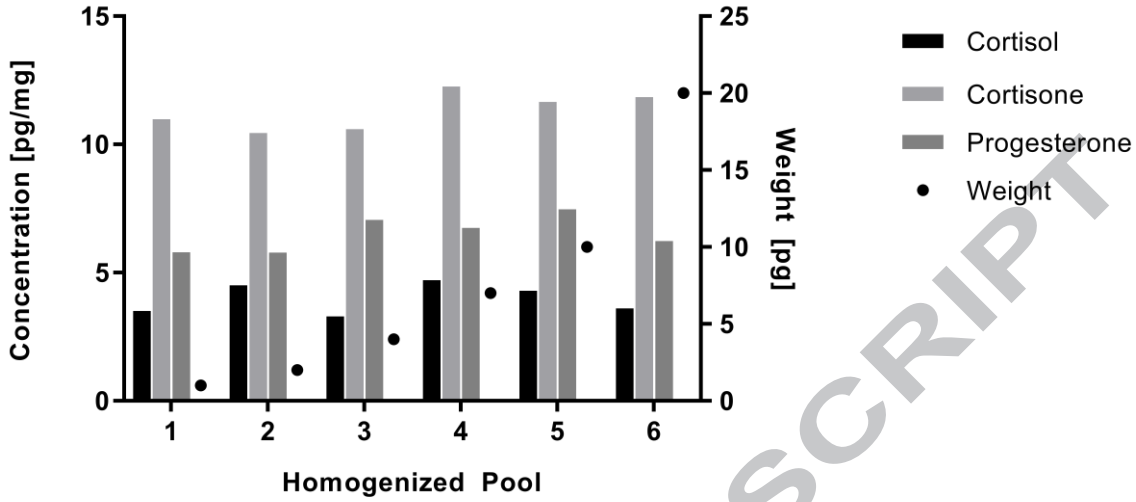

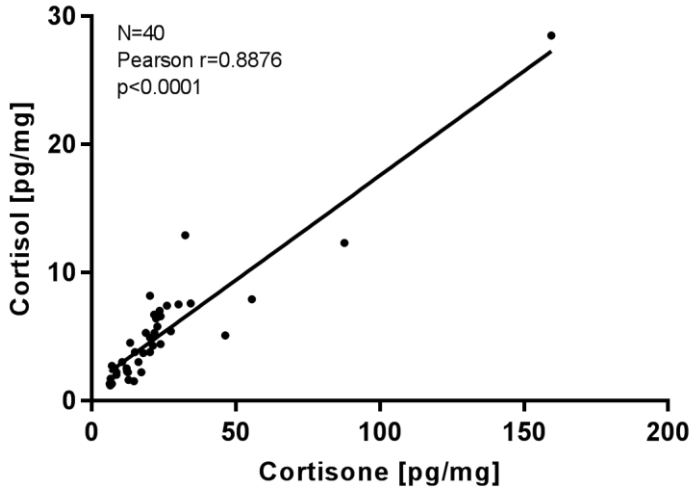

Highlights:

- An LC-MS/MS method was developed for the quantification of 12 steroid hormones in nails.
- 3 steroid hormones were detected in nails for the first time.
- Six steroid hormones were detected and quantified in human fingernails of mothers and their infants.
- No significant differences could be found between right and left hand steroid levels.